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Genetic diversity and *Wolbachia* infection of the *Drosophila* parasitoid *Leptopilina clavipes* in western Europe

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Abstract

***Wolbachia* are maternally transmitted bacteria that alter their arthropod hosts' reproduction in various ways, including parthenogenesis induction (PI). *Wolbachia*-induced parthenogenesis can have drastic effects on the genetic structure of its host because it potentially reduces populations to clones without genetic exchange. However, *Wolbachia*-induced parthenogenesis does not inevitably result in a reduction of genetic variation of infected populations vs. uninfected populations, because the parthenogenetic populations are initially derived from uninfected populations and can thus show similar genetic variation. Here we investigate these issues in infected and uninfected populations of the *Drosophila* parasitoid *Leptopilina clavipes* in western Europe. Wasps from 19 sites in the Netherlands, France and northern Spain were screened for *Wolbachia* and analysed using amplified fragment length polymorphism (AFLP) markers. All the populations from the Netherlands and mid-France were infected with the same two strains of *Wolbachia*, whereas populations from the Pyrenees were not infected. The infected and uninfected populations show identical levels of genetic variation, but have clearly diverged genetically, indicating the presence of a barrier that prevents gene flow. Within the infected wasps two distinct genotypes were found at multiple localities, indicating the coexistence of multiple clones. The conditions promoting clonal coexistence in *L. clavipes* are discussed.**

Keywords: AFLP, clonal coexistence, genetic diversity, *Leptopilina clavipes*, parthenogenesis, *Wolbachia*

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Introduction

Wolbachia bacteria are cytoplasmatic endosymbionts (α-proteobacteria) that infect a wide range of arthropod and nematode hosts. They are maternally inherited and enhance their transmission by altering the reproductive system of their host in various ways, i.e. cytoplasmic incompatibility (CI), male killing, feminization and parthenogenesis induction (PI) (Stouthamer *et al*. 1999). These alterations can have a profound impact on the genetic structure of their hosts, such as reduced gene flow between populations (Bordenstein 2003), reduced genetic variability (Plantard *et al*. 1998) and eventually speciation (Werren 1998; Bordenstein 2003). Although cytoplasmic incompatibility is the most widely distributed *Wolbachia*-induced reproductive alteration (O'Neill *et al*. 1997), the effects on the genetic structure of the host caused by parthenogenesis-inducing *Wolbachia* can be more drastic because it potentially reduces populations to clones without genetic exchange.

PI-*Wolbachia* are found mainly in the arthropod group Hymenoptera (Huigens & Stouthamer 2003), and sporadically in other groups, such as Coleoptera (Werren *et al*. 1995), Thysanoptera (Arakaki *et al*. 2001) and in mites of the genus *Bryobia* (Weeks & Breeuwer, 2001). PI-*Wolbachia* are restricted to hosts with haplodiploid modes of reproduction (Huigens & Stouthamer 2003) in which infected virgin females produce all-female offspring through gamete duplication (Stouthamer & Kazmer 1994; Gottlieb *et al*. 2002; Pannebakker *et al*. 2004). Gamete duplication results in completely homozygous offspring (Suomalainen *et al*. 1987) thereby further reducing genetic variation in infected populations.

However, *Wolbachia*-induced parthenogenesis does not necessarily imply a reduction in genetic variation compared

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to uninfected populations. The genetic variation between and within parthenogenetic populations mainly depends on their infection history. At the time of infection, the array of genotypes will be similar to the originally infected populations. Over time, mutation accumulation can cause the parthenogenetic populations to diverge from each other and from their sexual ancestors (Simon *et al*. 2003). Species in which both PI-*Wolbachia*-infected and -uninfected populations are known are ideal systems to study the consequences of PI-*Wolbachia* on the genetic structure of its host.

Leptopilina clavipes (Hymenoptera: Figitidae) is a parasitoid of *Drosophila* larvae occurring all over western Europe. Like other Hymenoptera, *L. clavipes* has haplodiploid sex determination. In the most common mode of reproduction (arrhenotoky), females develop from fertilized, diploid eggs and males from unfertilized, haploid eggs. Another haplodiploid mode of reproduction is parthenogenesis or thelytoky, in which all eggs develop into females. All *L. clavipes* populations known from northern Europe (i.e. the Netherlands, Denmark, Sweden) reproduce thelytokously (Nordlander 1980), which is *Wolbachia*-induced (Werren *et al*. 1995; Schidlo *et al*. 2002). In the taxonomic revision of the genus *Leptopilina*, Nordlander (1980) reported two male *L. clavipes* specimens from northern Spain (Gerona province) suggesting the presence of uninfected, arrhenotokous populations.

In the study reported here, we collected field samples from infected and uninfected populations in western Europe. Using amplified fragment length polymorphism (AFLP) markers, we investigated: (i) the relation between infected and uninfected populations of *L. clavipes*, (ii) the effects of PI-*Wolbachia* on the genetic variation of *L. clavipes* in Europe, and (iii) the existence of multiple clones among the infected populations. In addition, diversity of the *Wolbachia* bacteria was investigated by analysing sequence variation of the *Wolbachia*-specific *wsp* gene. This information can help to clarify the influence of PI-*Wolbachia* on the population genetic structure of its host.

Materials and methods

Field sampling

In the spring and summer of 2000, 2001 and 2002 *Leptopilina clavipes* populations across western Europe were sampled (sites from which parasitoids were collected are given in Table 1 and Fig. 1). In the Netherlands, the majority of the parasitoids were obtained by collecting fresh stinkhorn (*Phallus impudicus* Pers.) fruit bodies (Table 1). The fruit bodies were placed on moist vermiculite in plastic containers and left to decay on the forest floor for a period of 2 weeks. After 2 weeks the containers were taken into the laboratory where they were incubated at 20 ± 0.5 °C, L/D 16:8.

In southern Europe, the majority of the parasitoids were collected by setting traps that consisted of plastic containers with moist vermiculite baited with banana, cucumber or commercial mushrooms (*Agaricus bisporus*) and active

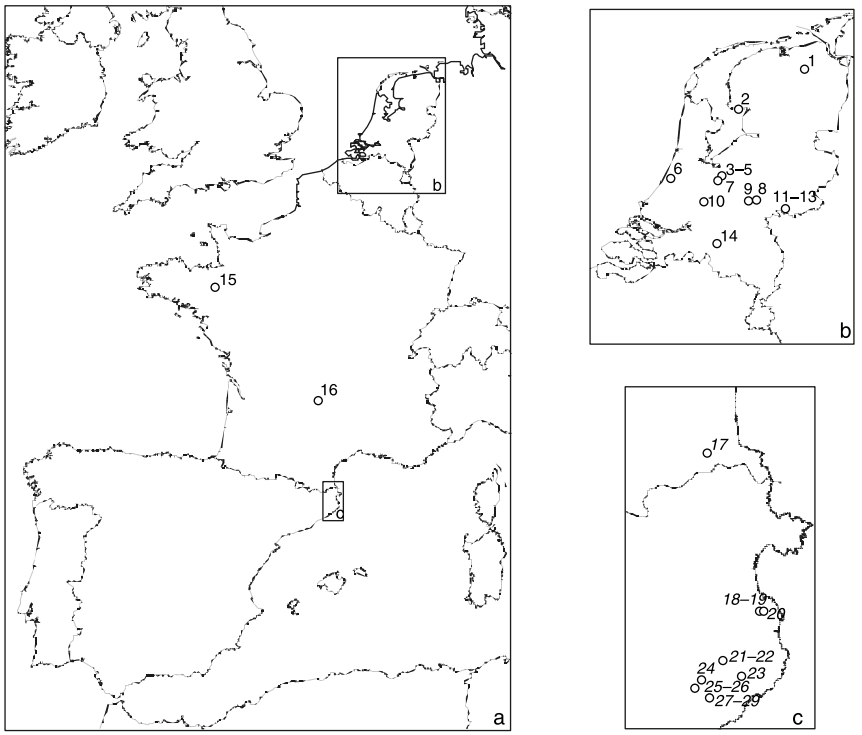

Fig. 1 Map of collection sites of *Leptopilina clavipes*. Collection sites in Europe (a), inserts showing collection sites in the Netherlands (b) and in Northern Spain and Southern France (c). Numbers represent site numbers as given in Table 1. Sites in italics indicate arrhenotokous populations, uninfected by *Wolbachia*.

Table 1 *Leptopilina clavipes* populations used in this study. Columns indicate population number (in order of latitude), population code, collection site, country and location of collection site, site coordinates, collection date, baits from which *L. clavipes* was reared (Ph., *Phallus impudicus*; B, banana; C, cucumber; M, commercial mushroom *Agaricus bisporus*; WM, unidentified wild mushroom), *Drosophila* species reared out of baits, number of iso-female lines set up and number of DNA extractions performed

No	Code	Site	Country	Location	Coordinates	Date	Bait	Drosophila species*	No. iso-female lines	No. DNA extractions
1	VOS	Vosbergen	NL	Eelde	N 53°13.02′ E 06°33.00′	2/9/00	Ph.	p†	2	1
2	EB2	Elfbergen	NL	Oudemirdum	N 52°51.00′ E 05°33.18′	23/6/00	Ph.	p†	5	3
3	DB17/9	Drakenburgh	NL	Baarn	N 52°13.02′ E 05°16.98′	17/9/99	B	—	1	2
4	DB23/9	Drakenburgh	NL	Baarn	N 52°13.02′ E 05°16.98′	23/9/99	B	—	1	2
5	DB00	Drakenburgh	NL	Baarn	N 52°13.02′ E 05°16.98′	2/6/00	Ph.	p†	4	2
6	WARMOND	Huys te Warmont	NL	Warmond	N 52°12.00′ E 04°30.00′	3/7/00	C	p,k,l,s,i,sim	1	2
7	HVLV	Hoge Vuursche	NL	Lage Vuursche	N 52°10.98′ E 05°13.98′	2/6/00	Ph.	p†	1	1
8	GBW	Groot Buunderkamp	NL	Wolfheze	N 52°00.00′ E 05°46.98′	15/6/00	C,M	p†	6	2
9	ONO	Oranje Nassau's Oord	NL	Wageningen	N 51°58.98′ E 05°42.00′	15/6/00	C,Ph.	p†	6	3
10	HOD	Hoog Oorsprong	NL	Doorwerth	N 51°58.98′ E 05°00.00′	15/6/00	Ph.	p†	6	3
11	KBH	Klein Beekermark	NL	s'Heerenbergh	N 51°55.38′ E 06°12.95′	14/6/00	Ph.	p†	6	3
12	BBH	Bergherbos	NL	s'Heerenbergh	N 51°54.28′ E 06°14.81′	14/6/00	Ph.	p†	7	3
13	MOH	Montferland	NL	s'Heerenbergh	N 51°54.17′ E 06°14.92′	14/6/00	C,Ph.	p†	7	4
14	DBK	De Blauwe Kei	NL	Oisterwijk	N 51°34.98′ E 05°12.00′	9/6/00	Ph.	p†	16	3
15	RENNES	Rennes	F	Rennes	N 48°04.98′ W 01°40.98′	13/7/01	Ph.	p	1	2
16	NEUVIC	Neuvic	F	Neuvic	N 45°22.98′ E 02°16.98′	15/7/01	Ph.	p	1	2
17	FLA	Larocque-des-Alberes	F	Larocque-des-Alberes	N 42°31.11′ E 02°55.83′	12/6/01	WM	p,b	na	1
18	TF	Torre Ferrana	E	El Montgrí	N 42°04.94′ E 03°08.11′	12/5/00	C,M	—	30	3

Table 1 *Continued*

No	Code	Site	Country	Location	Coordinates	Date	Bait	Drosophila species*	No. iso-female lines	No. DNA extractions
19	TF	Torre Ferrana	E	El Montgrí	N 42°04.94′ E 03°08.11′	14/5/01	C,M,B	p,k,b	4	2
20	DC	Duna Continental	E	El Montgrí	N 42°04.84′ E 03°08.66′	12/5/00	C,M	—	18	3
21	PDA	Puig de l'Avellana	E	Sant Sadurni de l'Heura	N 41°56.55′ E 02°59.36′	13/5/00	C,M,WM	—	11	3
22	PDA	Puig de l'Avellana	E	Sant Sadurni de l'Heura	N 41°56.55′ E 02°59.36′	14/5/01	C,M,B,WM	p,i,b	1	2
23	PLB	Puig de la Batteria	E	Calonge	N 41°53.85′ E 03°03.56′	14/5/01	C,M,B	p,k,b	3	1
24	PDLM2	Puig de les Miloques	E	Cassa de la Selva	N 41°53.26′ E 02°55.01′	14/5/01	C,M,B	p,l,b,i	na	1
25	CCAP	Can Capçanes	E	Cassa de la Selva	N 41°51.98′ E 02°53.07′	12/5/00	C,M,WM	—	2	2
26	CCAP	Can Capçanes	E	Cassa de la Selva	N 41°51.98′ E 02°53.07′	14/5/01	C,M,B	s,i,m	5	2
27	MOLL2	Molli d'en Llambi	E	Llagostera	N 41°50.59′ E 02°56.45′	12/5/00	C,M	—	10	2
28	MOLL1	Molli d'en Llambi	E	Llagostera	N 41°50.59′ E 02°56.46′	12/5/00	C,M	—	9	3
29	MOLL1	Molli d'en Llambi	E	Llagostera	N 41°50.59′ E 02°56.46′	13/5/01	C,M,B	i,b	3	1

*Abbreviations: p, *Drosophila phalerata*; k, *D. kuntzei*; l, *D. limbata*; s, *D. subobscura*; i, *D. immigrans*; b, *D. busckii*; m, *D. melanogaster*; sim, *D. simulans*.
†*D. phalerata* was the most abundant species in these traps. No individual flies were identified.

baker's yeast suspension (Table 1). To increase attractiveness to flies and parasitoids, 4.5 μg of (z)-11-octadecenyl acetate (dissolved in hexane), a major component of the *Drosophila* aggregation pheromone (Hedlund *et al*. 1996) was added. Traps were set on the forest floor and collected after 1 week, after which they were incubated in the laboratory as described above.

Traps from both regions were inspected twice a week for emerging parasitoids and flies for a period of 2 months. Emerging parasitoids were used to set up laboratory cultures, after which they were stored at –80 °C for subsequent DNA analysis. As a single female usually oviposits in more than one host larva per visited patch, wasps emerging from a single trap could be siblings. Furthermore, traps from one site were sometimes incubated together. Therefore, only one wasp per site was used in the AFLP analysis.

DNA isolation

DNA was extracted using a Nucleon BACC2 kit (Amersham Pharmacia Biotech) according to the manufacturer' s protocol. DNA was precipitated in absolute ethanol, washed with 70% ethanol after which the DNA was dissolved in 25 μL sterile water. When DNA was extracted from more than one individual per population (Table 1), one wasp was randomly selected to be included in the AFLP analysis.

Wolbachia *detection*

Infection status was determined by setting up multiple iso-female lines per site using *Drosophila phalerata* as a host at 20 ± 0.5 °C, 16:8 L/D and 65% relative humidity (Table 1). The sex ratio of the emerging offspring was scored and daughters from all-female broods were allowed to oviposit. When all-female broods were produced for the second time, they were classified as infected. Broods containing males were scored as uninfected.

Wolbachia infection was confirmed by amplifying the *Wolbachia*-specific *ftsZ* (Holden *et al*. 1993) and *wsp* (81F/691R primers; Zhou *et al*. 1998) genes. These and all other polymerase chain reactions (PCRs) were performed in a PTC-200 DNA engine (MJ Research). PCR conditions for the *ftsZ* gene were denaturation at 94 °C for 3 min, then 35 cycles of 94 °C for 45 s, 55 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 5 min. PCR conditions for the *wsp* gene were denaturation at 94 °C for 3 min, then 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 5 min. PCR products were visualized on a 1% agarose gel stained with ethidium bromide. Only after amplification of both genes was *Wolbachia* infection concluded.

To check for a correct amplification, the *wsp* PCR products were sequenced using ABI BigDye Terminator kit (Applied Biosystems), purified using Sephadex Autoseq G-50 Columns (Amersham Pharmacia Biotech) and visualized on an ABI 377 DNA Sequencer (Applied Biosystems). Sequences were deposited at GenBank (Accession nos AY429025–AY429040).

AFLP analysis

The genetic diversity of *L. clavipes* was analysed using the AFLP technique (Vos *et al.* 1995). For each individual, 7 μL of DNA was restricted with *Eco*RI and *Mse*I and ligated with T4 DNA ligase (New England Biolabs) to adaptors at 37 °C for 2 h, after which the ligase was inactivated by heating for 10 min at 65 °C. The restriction–ligation mixture was used as a template in the preselective PCR, using *Mse* + A and *Eco* + C primers and the AFLP Core Mix (Applied Biosystems) in a total reaction volume of 15 μL. Preselective PCR was carried out under the following conditions: 72 °C for 2 min, 94 °C for 2 min followed by 20 cycles of 94 °C for 5 s, 56 °C for 30 s and 72 °C for 2 min. Prior to the selective PCR, the preselective mixture was diluted 1:10 in Tris-EDTA (TE) buffer. The selective PCR was performed with primers similar to those used in the preselective reaction, but with extra bases at the 3'-end. Based on their high level of polymorphism two primer combinations (*Mse*-CA with the fluorescently labelled *Eco*-ACA and *Eco*-AGG) were used in the selective PCR amplification. For selective PCR amplification, a touchdown reaction was used with 94 °C for 5 s, 65 °C for 30 s and 72 °C for 2 min, followed by eight cycles in which the annealing temperature was lowered to 56 °C in 1 °C steps. This was followed by 24 cycles at an annealing temperature of 56 °C and an extension at 72 °C for 35 min.

PCR products were visualized on 5% Long Ranger polyacrylamide gels (BMA Bio Products) running on an ABI 377 DNA Sequencer (Applied Biosystems). Data were processed using GENESCAN v3.1 (Applied Biosystems). Samples were manually checked for correct alignment of the size standard and corrected if necessary. The fluorescent profiles were imported into GENOGRAPHER v1.60 (J.J. Benham; http://hordeum.oscs.montana.edu/genographer) and presence or absence of fragments was scored between 50 and 500 bp.

Analysis

The presence or absence of fragments was coded in a matrix after which the pairwise genetic distances were calculated using Nei and Li's index (Nei & Li 1979) in TREECON for Windows v1.3b (Van de Peer & De Wachter 1994). UPGMA cluster analysis was performed to visualize the genetic distances in a tree format. The presence/absence matrix was also used to perform principal components analysis (PCA). This and all other statistical analyses were carried out using R version 1.5.1 (Ihaka & Gentleman 1996).

The pairwise genetic distance matrix calculated by TREECON was used to test for the correlation between genetic and geographical distances by a Mantel test (Mantel 1967).

Results

A total of 88 sites in western Europe were sampled in the seasons 2000–02, of which 19 yielded *Leptopilina clavipes* (Fig. 1, Table 1). *Wolbachia* infection was detected in all the populations from the Netherlands and in two populations from mid-France. All populations south of the Pyrenees and the only population from the foothills of the Pyrenees in France were uninfected. Traps from northern Europe yielded *Drosophila phalerata*, *D. kuntzei*, *D. limbata*, *D. subobscura*, *D. immigrans* and *D. simulans*; traps from southern Europe yielded *D. phalerata*, *D. kuntzei*, *D. limbata*, *D. subobscura*, *D. immigrans*, *D. busckii* and *D. melanogaster* (Table 1). Of the 88 sampled sites, 42 were located in the area between mid-France and the Pyrenees. Sampling in this area yielded complete *Drosophila* and parasitoid communities, except for *L. clavipes* (data not shown).

The *wsp* fragments amplified from the infected populations were 485-bp long and had only one polymorphic nucleotide at position 303, which was polymorphic for A/T in all 16 samples. Because *wsp* is a single copy gene (Braig *et al*. 1998), this polymorphism most likely represents two different *Wolbachia* strains. However, the low diversity at the *wsp* locus suggests that all the northern populations are infected by the same two strains of *Wolbachia*.

The AFLP analysis based on two primer combinations yielded a total of 142 scorable fragments from *L. clavipes*, of which 69 (49%) were polymorphic and present in more than one individual. In general, a low genetic distance was found both within and between the modes of reproduction (Fig. 2). However, there is clear separation of the arrhenotokous and thelytokous *L. clavipes* populations, as can be seen from both the UPGMA tree (Fig. 2) and the PCA analysis (Fig. 3). The genetic distance between the two modes of reproduction is smaller than the genetic distance to and between the two out-groups (Fig. 2).

In addition to separation of arrhenotokous and thelytokous populations, PCA analysis indicates a split in the thelytokous populations (Fig. 3, PC1–PC2 plot). Group T2 includes wasps of five Dutch samples (VOS-1, DB17/9-3, DB23/9-4, WARMOND-6 and BBH-12) from four different locations. All other samples are in group T1, including

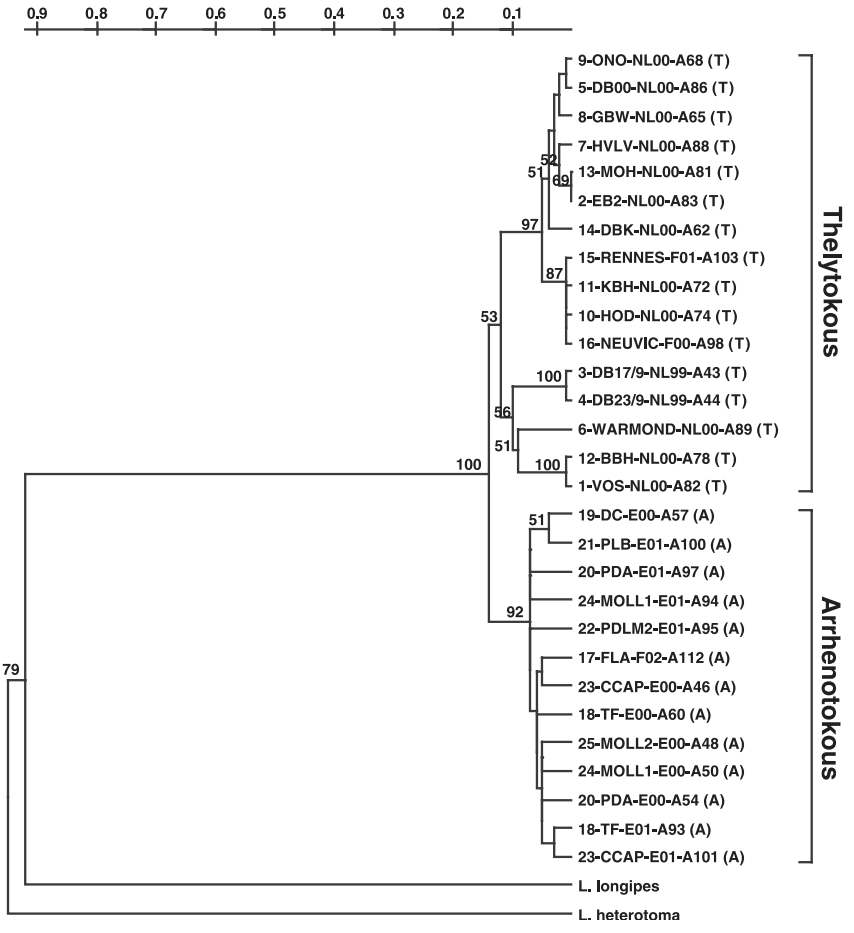

Fig. 2 UPGMA tree for genetic distance of 13 arrhenotokous and 16 thelytokous populations. Scale indicates genetic distance (Nei & Li 1979). Numbers at nodes represent bootstrap values over 50% (1000 replicates). Codes represent population number, population code, country, collection year, DNA isolation number and reproductive mode in brackets (see also Table 1).

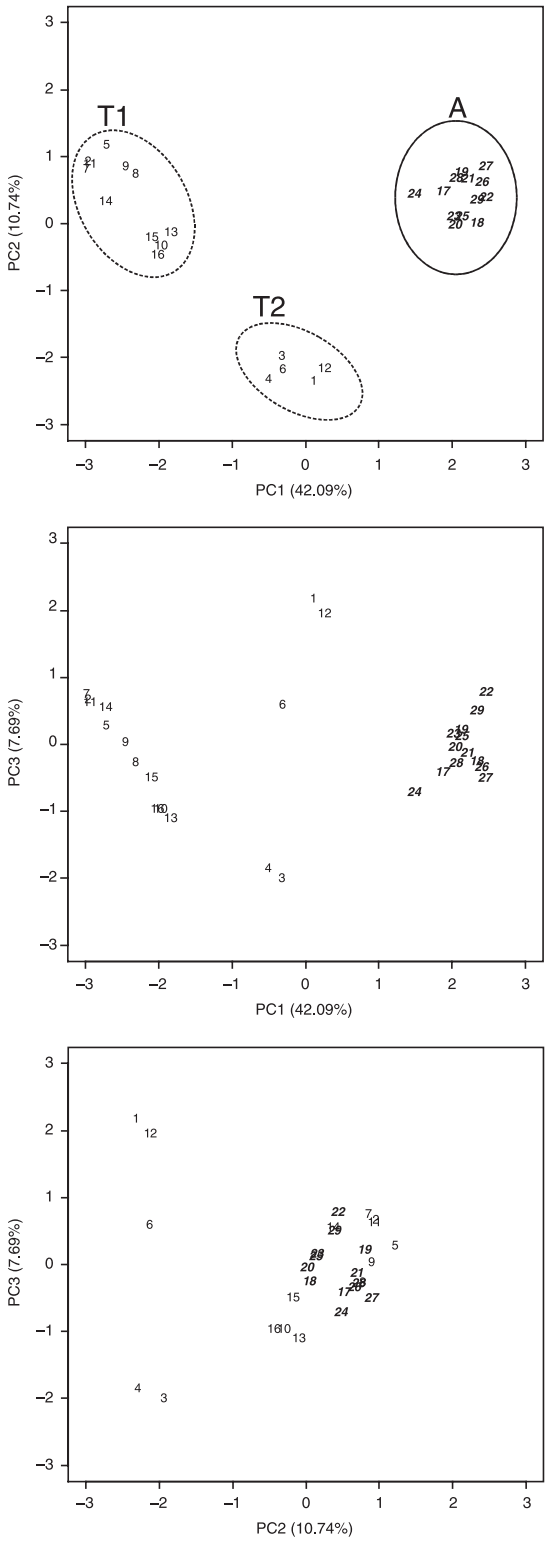

Fig. 3 Plot of the first three principle components that describe 60.52% of the AFLP variation in *Leptopilina clavipes*. Numbers represent site numbers as given in Table 1. Bold italic numbers represent arrhenotokous populations, nonitalic numbers represent thelytokous populations. Broken line circles indicate thelytokous groups T1 and T2, solid line circle indicates arrhenotokous group A.

wasps from the same sample sites as present in group T2 (e.g. DB00-5), but collected a year later.

The mean pairwise genetic distance between all thelytokous populations (0.077, SD = 0.042) was larger than that between all arrhenotokous populations (0.067, SD = 0.015) (*t*-test assuming unequal variance, $t_{162} = -2.514$, $P = 0.013$). Because of large differences in the sample scale of arrhenotokous and thelytokous populations (maximum distances 75 and 924 km, respectively), the comparison was repeated with a subset of the thelytokous populations (maximum distance 75 km). On this scale, the thelytokous populations had a mean pairwise genetic distance of 0.076 (SD = 0.041) which was not different from the arrhenotokous populations (*t*-test assuming unequal variance, $t_{67} = -1.648$, $P = 0.104$).

Mantel tests for correlations between genetic and geographical distance were performed separately for arrhenotokous and thelytokous samples. For both arrhenotokous and thelytokous samples no correlation between genetic and geographical distance was found ($n = 9, r = -0.072$, $P = 0.6184$ and $n = 16$, $r = -0.138$, $P = 0.7153$, respectively).

Discussion

All populations from the Netherlands and the two mid-France populations are infected by *Wolbachia*, which renders them thelytokous. Populations sampled in northern Spain are arrhenotokous and free of *Wolbachia*. The low genetic distance between the infected and uninfected populations indicates a single ancestral type. Nevertheless, there is a clear separation between the two modes of reproduction, coinciding with a separation between northwestern European and Iberian populations.

A comparison of genetic distances between the two groups relative to the distances to and between the outgroups, suggests that the uninfected and infected populations are populations of the same species. Morphological data confirm this, on the basis of which Nordlander (1980) placed two males from northern Spain (L'Estartit) in the same species as all-female populations from northern Europe. Furthermore, experiments with males derived from thelytokous populations by antibiotic elimination of the *Wolbachia* bacteria, showed that these are able to produce viable offspring when mated to arrhenotokous females (B.A. Pannebakker *et al.* unpublished results). Nevertheless, the two groups of populations have diverged genetically and the division between the two groups implies the presence of a profound barrier to gene flow between them.

A factor potentially reducing gene flow between both reproductive modes is their phenology (i.e. temporal isolation). The Spanish samples were collected in the first half of May, whereas the northern samples were collected from the beginning of June onwards, until the last week of September. The season for *Leptopilina clavipes* appears to begin earlier in Spain, favoured by the humid spring conditions. Mushrooms

are available in winter and early spring, but during the dry summer months, the mushrooms density decreases considerably (E. Gracia, pers. commun.). Only after substantial rainfall in autumn (October–November), are the mushrooms and fungivorous *Drosophila* species present again.

The phenology of a *L. clavipes* population in the Netherlands was described by Driessen *et al*. (1990). It starts in the beginning of June, when the population shows a steep increase and forms a first peak coinciding with a peak in the fruiting of *Phallus impudicus* which is the major food source of *Drosophila phalerata*, the natural host species of Dutch *L. clavipes* populations. After that, the population decreases over the summer months to form a second peak in mid-September coinciding with a second peak in mushroom density.

The proposed phenological division between the two groups appears to be counteracted by collection of the southern France sample from Larocque-des-Alberes (FLA-17) in mid-June. It should be noted, however, that this sample contained only one individual and thus is likely to be a remnant from the declining spring peak.

A second factor enhancing genetic divergence between the reproductive modes is spatial isolation. Despite extensive sampling efforts over three successive field seasons (2001–03), no area was found where infected and uninfected *L. clavipes* coexist. The sample from Larocque-des-Alberes (FLA-17) was the only *L. clavipes* sample collected in Mediterranean France. Apart from *L. clavipes*, the region between the Pyrenees and the Massif Central in France (Languedoc-Roussillon) harbours a complete *Drosophila* and parasitoid community (B.A. Pannebakker *et al*. unpublished data). Hence, there appears to be a gap between the infected populations on the Massif Central in France (e.g. NEUVIC-16) and the uninfected populations. The reasons for such a disjunct distribution are unknown, but similar patterns are known for other insects such as some Geometrid moth species (Mironov 2003).

A third factor potentially reducing gene flow between the two reproductive modes is the infection of the northern populations with PI-*Wolbachia*. Being parthenogenetic, the females no longer need to fertilize their eggs to produce female offspring. However, in areas where infected and uninfected populations overlap, matings could occur between them. Although uninfected arrhenotokous *L. clavipes* males readily mate with infected thelytokous females, their sperm is not used for fertilization (B.A. Pannebakker *et al*. unpublished results). In natural infected populations, males occur very rarely (Driessen *et al*. 1990), presumably due to inefficient transmission of the *Wolbachia* bacteria. As these males can produce viable offspring when mated to arrhenotokous females (B.A. Pannebakker *et al*. unpublished results), some gene flow could occur from infected to uninfected populations if they were to overlap in distribution and phenology. Hence, PI-*Wolbachia*, at least potentially, can cause unidirectional reproductive isolation between the two groups. To some extent unidirectional mating incompatibilities alone can already be responsible for genetic divergence between populations, as is shown by CI-*Wolbachia* research (Bordenstein 2003). Hence, the arrhenotokous populations are isolated from the thelytokous ones by phenology, geography and the presence of PI-*Wolbachia* in the thelytokous populations.

In addition to their role in the division between the infected and uninfected populations, PI-*Wolbachia* also affect the genetic diversity between the infected populations. In general, PI-*Wolbachia* can be expected to have contrasting effects on genetic diversity at different levels. On the individual level, the creation of fully homozygous offspring through *Wolbachia*-induced gamete duplication (Suomalainen *et al*. 1987) leads to a reduction in genetic diversity. At the population level, however, the infectious nature of *Wolbachia*-induced parthenogenesis (horizontal transmission) initially leads to clonal genotypes similar to those of the uninfected ancestral populations (Simon *et al*. 2003).

In *L. clavipes*, both the cluster analysis and the PCA show two distinct groups within the *Wolbachia*-infected wasps. Most samples, including the two infected French samples RENNES-15 and NEUVIC-16 present one large group (T1). Five Dutch samples group together in a smaller group (T2). Repeated analysis of the samples in group T2 excludes PCR errors as the cause for this division. It is more likely that the division within the infected *L. clavipes* is due to the presence of at least two dominant clonal genotypes in northwestern Europe.

In general, most clonal diversity arises from multiple transitions to parthenogenesis, rather than from mutation accumulation within parthenogenetic lineages (Vrijenhoek 1998). One way to obtain multiple transitions is by horizontal transmission of *Wolbachia* by means of superparasitism (Huigens *et al*. 2000). Another consequence of horizontal transmission in the initial stages of the infection, i.e. when populations consist of infected and uninfected individuals, is the association of the same *Wolbachia* strains with multiple host genotypes. Horizontal transmission is likely to occur in *L. clavipes* where superparasitism in the field is common (Driessen & Hemerik 1991) and could have resulted in the association of the same two *Wolbachia* strains in both clonal genotypes and all the northern populations.

The clonal genotypes are not associated with certain localities, but coexist in at least two of the sampled localities. Because of our sampling methods based on oviposition traps, we effectively sampled only one female per population, thereby increasing the chance of randomly selecting one of the two dominant clones. However, at locations that were sampled in more than one season (e.g. DB17/9-3, DB23/9-4 and DB'00-5 at Drakenburgh) the two clones were collected in subsequent years. Furthermore, at BBH-12 and MOH-13, locations that are less than 1 km apart, the two clones were collected at the same date. More intensive

sampling at a smaller scale will probably yield multiple clonal genotypes at more locations.

Horizontal transmission is an important factor in the origin of PI-*Wolbachia*-associated clonal diversity (Plantard *et al*. 1998; Simon *et al*. 2003). However, for the maintenance of clonal diversity in general, several other mechanisms have been suggested. Occasional sexual reproduction as a means of generating new clonal genotypes (Parker 1979; Weeks & Hoffmann 1998) can be excluded in *L. clavipes*, as infected females do not use the sperm when mated to uninfected males (B.A. Pannebakker *et al.* unpublished data). Another possibility explaining the coexistence of multiple clones is likely to hold for our *L. clavipes* data. In our study, genetic diversity was estimated using the AFLP method. The AFLP method generates markers from across the entire genome (Vos *et al*. 1995; Sharbel 1999) and consequently a genotype is constituted from both coding and noncoding DNA. As selection acts only on traits that are adaptive in a certain environment, a difference in genotype does not necessarily imply a difference in phenotype. In our data no indication for phenotypical differences between the two clones could be found. On the contrary, both clones were widespread and collected in similar habitats using the same array of baits. Co-existence of multiple clones without apparent phenotypical differences was also reported for the earthworm *Octolasion tyrtaeum* (Jaenike *et al*. 1980). In the absence of spatial heterogeneity, coexistence of multiple clones with nearly identical niches can be stable only when they subdivide their habitat ecologically. Otherwise, the coexistence is likely to be temporary, as one clone will eventually replace the others through competitive exclusion or clonal drift (Jaenike *et al*. 1980).

It is likely that drift processes dramatically reduced the array of clones that must initially have been generated by *Wolbachia*, to the low number of widespread clones present in western Europe today. If the differences in genotype between these widespread clones in *L. clavipes* are assumed to be neutral, their coexistence is temporary and drift will eventually result in the dominance of one single clonal genotype. However, due to human activity western European forests are highly fragmented (European Environment Agency 2002) and form islands within the cultural landscape. As a parasitoid specializing on fungal breeding *Drosophila* (Driessen *et al*. 1990, 1991), *L. clavipes* depends on forest habitats and is thus likely to have a metapopulation structure (Hanski 1999). Local extinction by drift can cause the disappearance of one of the clonal genotypes at a particular locality, while at other localities the other genotype can disappear. Together with the probability of recolonization, these processes make the probability of extinction of one of the clones from the metapopulation extremely small.

To conclude, the *Wolbachia*-infected and -uninfected *L. clavipes* populations in western Europe are genetically separated, but are still a single species. The genetic division probably reflects a physical division as no coexistence of the two modes of reproduction was found. Similar genetic variation exists in infected and uninfected populations, which is due to the existence of multiple clones within the infected populations. The factors enabling this clonal coexistence are still uncertain and will be subject of further investigation.

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